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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

		Attorney Docket No. 02043
		U.S Application No. (if known, see 37 CFR 1.5) 10/069950
INTERNATIONAL APPLICATION NO. PCT/FR00/02523	INTERNATIONAL FILING DATE September 13, 2000	PRIORITY DATE CLAIMED September 14, 1999

TITLE OF INVENTION
COMPOSITION TO BE ADMINISTERED THROUGH MUCOUS MEMBRANE

APPLICANT(S) FOR DO/EO/US
Sophie Gaubert and Rene Laversannne

Applicant herewith submits to the United States Designated Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3).
   a. ☐ are transmitted herewith (only if not required by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5).

Items 11 to 16 below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ As assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:

23338
PATENT TRADEMARK OFFICE

17. ☒ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a)(1)-(5):		
Neither international preliminary examination fee (37 CFR 1.482) Nor international search fee (37 CFR 1.445(a)(2) paid to USPTO And International Search Report not prepared by EPO or JPO..... $1,040.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by EPO or JPO.....$890.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International search fee (37 CFR 1.445(a)(2)) paid to USPTO..... $740.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) But all claims did not satisfy provisions of PCT Article 33(1)-(4).....$710.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) And all claims satisfied provisions of PCT Article 33(1)-(4)..... $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$890.00	
Surcharge of **$130.00** for furnishing oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	19 -20=		X $18.00	$	
Independent Claims	4 -3=	1	X $84.00	$84.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)				$	
TOTAL OF ABOVE CALCULATIONS =				$974.00	
Reduction of ½ for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$974.00	
Processing fee of **$130.00** for furnishing English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f).				$	
TOTAL NATIONAL FEE =				$974.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31).				$40.00	
TOTAL FEES ENCLOSED =				$1014.00	
				Amount to be refunded:	$
				charged:	$

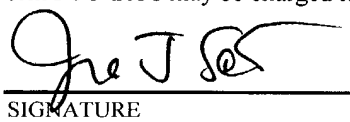
a. ☐ A check in the amount of $ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 04-0753 in the amount of $ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☐ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-0753. A duplicate copy of this sheet is enclosed.

d. ☒ A payment of $ 1014.00 is made by credit card. A Credit Card Payment Form (PTO-2038) is attached hereto. The Commissioner is hereby authorized to charge payment of any additional filing fees required under 37 CFR 1.16 or any patent application processing fees under 37 CFR 1.17, or credit any over payment to the credit card account shown on the attached Credit Card Payment Form. Refund of all amounts overpaid, including those of twenty-five dollars or less, is specifically requested. Any fees not accepted by the credit card shown on Form PTO-2038 may be charged to Deposit Account No. 04-0753.

SEND ALL CORRESPONDENCE TO:
Dennison, Scheiner & Schultz
612 Crystal Square 4
1745 Jefferson Davis Highway
Arlington, VA 22202-3417
Telephone (703) 412-1155 Ext. 23
Facsimile (703) 412-1161



SIGNATURE
Ira J. Schultz
NAME
28666
REGISTRATION NUMBER

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JC13 Rec'd PCT/PTO 07 MAR 2002

Dkt. 02043

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of: Group Art Unit:

SOPHIE GAUBERT et al Examiner:

Serial No.: US National Phase of PCT/FR00/02523

Filed: concurrently herewith

For: COMPOSITION TO BE ADMINISTERED THROUGH MUCOUS MEMBRANE

PRELIMINARY AMENDMENT AND INFORMATION DISCLOSURE STATEMENT

Honorable Assistant Commissioner for Patents
Washington, DC 20231

Sir:

Before calculation of the filing fee, please amend the above-identified application as follows:

IN THE CLAIMS:

Please amend the claims as set forth hereinbelow and in the attached appendix:

16. (Amended) A method of treating a human or animal body by vaccination, comprising mucosal administration [via the mucosa] of a composition comprising multilamellar vesicles with an onion-like structure having an internal liquid crystal structure formed by a stack of concentric bilayers based on amphiphilic agents alternating with layers of water, an aqueous solution or a solution of a polar liquid, and into

which at least one antigen is incorporated.

17. (Amended) A method according to claim 16, said administration is carried out nasally.

18. (Amended) A method of producing antibodies, comprising introducing into a host organism, via mucosa, lamellar vesicles with an onion-like structure having an internal liquid crystal structure formed by a stack of concentric bilayers based on amphiphilic agents alternating with layers of water, an aqueous solution or a solution of a polar liquid and into which at least one antigen is incorporated, then removing and purifying said antibodies.

19. (Amended) A method of producing IgA, comprising introducing into a host organism, via mucosa, lamellar vesicles with a multilamellar onion-like structure with an internal liquid crystal structure formed by a stack of concentric bilayers based on amphiphilic agents alternating with layers of water, an aqueous solution or a solution of a polar liquid and into which the appropriate antigen is incorporated, and removing and purifying said immunoglobulins.

Please cancel claims 1-15 without prejudice or disclaimer of the subject matter thereof, and add the following new claims:

--20. (New) A method of treating a human or animal body comprising introducing into the body mucosally a composition

comprising lamellar vesicles with a multilamellar onion-like structure with an internal liquid crystal structure formed by a stack of concentric bilayers based on amphiphilic agents alternating with layers of water, an aqueous solution or a solution of a polar liquid and into which the appropriate antigen is incorporated, and removing and purifying said immunoglobulins.

21. (New) Method according to claim 20, wherein said composition is a composition induces a mucousal response.

22. (New) Method according to claim 20, wherein said composition is a composition induces a systemic seric response.

23. (New) Method according to claim 20, wherein said composition induces production of antibodies.

24. (New) Method according to claim 20, wherein said composition is a vaccine which induces protection of the human or animal against an infection for which said antigen is responsible.

25. (New) Method according to claim 24, wherein said antigen is of exogenous or intrinsic natural origin.

26. (New) Method according to claim 20, wherein said antigen is selected from the group consisting of:

proteins and glycosylated proteins,

peptides,

lipopeptides,

polysaccharides, and

mixtures thereof.

27. (New) Method according to claim 20, wherein said vesicles contain at least one surfactant selected from the group consisting of:

phospholipids,

hydrogenated phospholipids,

linear or branched, saturated or mono- or poly-unsaturated C_6 to C_{30} fatty acids or an alkali, alkaline earth or amine salt thereof,

esters or ethoxylated esters of said fatty acids with saccharose, sorbitan, mannitol, glycerol, polyglycerol or glycol,

mono-, di- or triglycerides or mixtures of glycerides of said fatty acids,

linear or branched, saturated or mono- or poly-unsaturated C_6 to C_{30} fatty alcohols and ethoxylated linear or branched, saturated or mono- or poly-unsaturated C_6 to C_{30} fatty alcohols,

ethers of said fatty alcohols and saccharose, sorbitan, mannitol, glycerol or polyglycerol, or glycol,

polyethoxylated vegetable oils, and hydrogenated polyethoxylated vegetable oils,

block polymers of polyoxyethylene and polyoxypropylene (poloxamers),

polyethyleneglycol hydroxystearate,

alcohols with a sterol skeleton,

sphyngolipids,

polyalkylglucosides,

copolymers of polyethylene glycol and alkylglycol, and

di- or tri-block copolymers of ethers of polyethyleneglycol and polyalkyleneglycol.

28. (New) Method according to claim 20, wherein said vesicles also contain at least one co-surfactant which improves rigidity and/or tightness of the membranes of said vesicles.

29. (New) Method according to claim 28, wherein said co-surfactant is selected from the group consisting of:

cholesterol, cholesterol derivatives and cholesterol esters,

derivatives with a sterol skeleton, and sterol skeleton derivatives of plant origin, and

ceramides.

30. (New) Method according to claim 20, wherein said vesicles also contain an immuno-modulating substance.

31. (New) Method according to claim 20, wherein said vesicles have diameter of said vesicles is in the range 0.1 µm

to 25 μm.

32. (New) Method according to claim 20, wherein the bilayers of said vesicles comprise at least two surfactants, one of said surfactants having a hydrophilic-lipophilic balance (HLB) in the range 1 to 6, and the other having a hydrophilic-lipophilic balance (HLB) in the range 3 to 15.

33. (New) Method according to claim 20, wherein antigen in the vesicles have an encapsulation yield of more than 50%.

34. (New) Method according to claim 20, wherein said administration is nasal administration.--

REMARKS

The claims have been amended to delete use claims 1-15, and to generally place the claims in better form for US practice. The subject matter of claim 1 was incorporated into claim 15 to make that claim independent. In addition, new claims 20-34 have been added which correspond to the subject matter of canceled claims 1-15.

Attached is the search report of the corresponding PCT application, together with copies of the references cited therein, which are listed on the attached Form PTO-1449. In addition to the listed references, attention is also directed to pending US application Serial No. 09/536,153 filed March 28, 2000, which claims the priority of FR 97/12085, corresponding to FR 2,769,022 listed on the Form PTO-1449.

Respectfully submitted,

Ira J. Schultz
Registration No. 28666

APPENDIX

16. (Amended) A method of treating a human or animal body by vaccination, [characterized in that it comprises] comprising mucosal administration [via the mucosa] of a composition [as defined in any one of claims 1 to 15] comprising multilamellar vesicles with an onion-like structure having an internal liquid crystal structure formed by a stack of concentric bilayers based on amphiphilic agents alternating with layers of water, an aqueous solution or a solution of a polar liquid, and into which at least one antigen is incorporated.

17. (Amended) A method according to claim 16, [characterized in that] said administration is carried out nasally.

18. (Amended) A method of producing antibodies, [characterized in that it comprises] comprising introducing into a host organism, via [the] mucosa, lamellar vesicles with an onion-like structure having an internal liquid crystal structure formed by a stack of concentric bilayers based on amphiphilic agents alternating with layers of water, an aqueous solution or a solution of a polar liquid and into which at least one antigen is incorporated, then removing and purifying said antibodies.

19. (Amended) A method of producing IgA, [characterized

in that it comprises] comprising introducing into a host organism, via [the] mucosa, lamellar vesicles with a multilamellar onion-like structure with an internal liquid crystal structure formed by a stack of concentric bilayers based on amphiphilic agents alternating with layers of water, an aqueous solution or a solution of a polar liquid and into which the appropriate antigen is incorporated, and removing and purifying said immunoglobulins.

10/069950
JC13 Rec'd PCT/PTO 07 MAR 2002

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COMPOSITION TO BE ADMINISTERED THROUGH MUCOUS MEMBRANE

The present invention relates to novel compositions for administration via the mucosa. More particularly, it relates to a pharmaceutical composition, in particular a vaccine composition for administration via the mucosa.

It also relates to a method for producing antibodies, in particular IgA.

A number of definitions are given below:

Administration via the mucosa: non-invasive administration of an antigen to a mucous site, for example:

- naso-pharyngeal area;
- buccal area;
- bronchial tree;
- intestine;
- uro-genital tract;
- inner ear;
- conjunctiva;
- mammary, salivary and lachrymal glands.

Of these different constituents of mucus associated lymphoid tissue (MALT), certain introduction routes are more readily accessible and more readily acceptable: nasal, buccal, gastro-intestinal, rectal and vaginal.

Mucous response: The response of the immune system in the mucosa, characterized by production of IgA (isotype in high concentrations in the mucosa) and IgG and/or a cellular response in the mucous site and lymph nodes.

Systemic response: The response of the generalized immune system resulting from the presence of circulating antibodies (IgG and IgA) and/or a cellular response (T helper or CTL) in the secondary lymphoid organs (spleen, nodes).

Dissemination of response: Installation of a mucous response in compartments other than that used for introduction, because of the circulation and relocalization of lymphocytes induced at the

administration site (vaginal IgA after nasal administration, for example).

Adjuvant: The substance added to the antigen to amplify and orientate the specific immune response of that antigen.

Synthetic vector/vectorization: Incorporation of the antigen into or on the surface of a particle or vesicle, to protect the antigen and/or facilitate its capture by competent cells of the immune system and/or to facilitate presentation of the antigen by said cells, and to amplify the immune response. The addition of an empty vector to the free antigen provides little or no amplification.

Until recently, vaccines were produced from dead, attenuated or less virulent micro-organisms. Today's pharmaceutical industry seeks to avoid such an approach, to limit secondary effects, to facilitate production, for safety in administration and for efficacy reasons.

Advances in molecular biology have resulted in the industrial production of sub-units of such micro-organisms, in particular proteins that are termed recombinant proteins, either membrane, nuclear or cytoplasmic proteins. Unfortunately, such sub-units are not in themselves sufficiently immunogenic to supply a sufficient response to vaccination.

They have to be supplemented with adjuvants or vectorized to induce a sufficient response.

Today, the only adjuvants that are acceptable for human medicine are aluminum hydroxide or phosphate, also calcium phosphate. These salts are in the form of a suspension of grains of the aluminum or calcium salt, onto the surface of which the antigen is adsorbed. They have a number of disadvantages: they induce local inflammation reactions and the production of IgE, they are not effective for all antigens and they are incapable of causing CTL type cellular mediation reactions. Thus,

until now they have not been used for administration via the mucosa.

Mucous surfaces are very important, firstly because they are present in all tracts and secondly, because they are the first line of defense against the invasion of pathogenic agents.

Mucous surfaces are protected both by innate or non adaptive defense mechanisms (peristalsis, cilia movements and mucous) and by triggering a cellular immune response and an adaptive humoral response specific to the pathogenic agent, which can be generalized to other lymphoid organs. Mucus associated lymphoid tissue (MALT) is responsible for the specific component.

In this respect, vaccination via the mucosa represents a considerable advantage.

Currently, all vaccines except that for poliomyelitis are injectable, which involves:

- medicalization;
- low acceptance;
- discomfort (fever, injection site pain,...);
- risks of contamination (HIV, hepatitis);
- high costs in developing countries.

Administration via the mucosa would be of advantage for a variety of reasons:

- production costs would be lower due to the less stringent preparation conditions compared with those for an injectable vaccine;
- its non invasive nature, which eliminates problems linked to injection mentioned above.

To this end, it must:

- provide immunity at the mucous administration sites, to strengthen the mucous barrier by dint of a specific response, providing an advantage for infections that can be transmitted via the mucosa (respiratory, genital,...);

- provide systemic immunity and an effective generalized response following easy administration;
- not cause secondary effects (local irritation).

A fairly complete review of mucosal immunology can be found in the "Handbook of Mucosal Immunology" edited by Pearay Ogra et al., Academic Press, Inc., San Diego, California, USA.

A number of trials and studies have been carried out with a view to developing novel adjuvants and vectors that can induce a mucous and systemic response that is effective as regards protection against the pathogenic agent. Different studies concern the use of live recombinant vectors (attenuated bacteria or viruses), synthetic vectors (immunostimulating complexes also known as iscoms, an abbreviation of the expression "immunostimulating complexes", liposomes, microspheres...) or micro-organism toxins (cholera toxin (CT), or heat labile *E. Coli* toxin, (LT)). However, a large proportion of these studies are still in the development stage and the majority of formulations have the following disadvantages:

- high toxicity (ST and LT), and thus are not possible for human application;
- difficulties in preparation (microspheres);
- low stability (liposomes);
- low efficacy (liposomes).

A number of patents concern administration via the mucosa of antigens that are vectorized or supplemented by different systems. The following can be cited:

- European patent EP-A-0 440 289 (Duphar), which describes the use of liposomes mixed with an antigen for vaccination against influenza. The lipids act as an adjuvant, the same result being obtained when the antigen is mixed extemporaneously with empty liposomes;

- International patent application WO-A-98/10748 (The School of Pharmacy), using cationic liposomes, in the case of injection or of administration via the mucosa;
- United States patent US-A-5 679 355 (Proteus), describing non-ionic vesicles, which can be administered parenterally or via the mucosa (especially orally).

Further, a great deal of scientific literature describes the use of polymer microparticles as a mucous membrane antigen vector. A good review of that literature can be found in: D. T. O'HAGAN, Adv. Drug. Deliv. Rev., **34** (1198), 305-320.

However, none of the above technologies has yet culminated in a commercially available product. Antigen presentation methods must, therefore, be further improved and the development of novel adjuvants or antigen vectors remains an urgent need in the vaccine industry.

Vesicles, spherical objects formed by a molecular arrangement of amphiphilic molecules, include mulilamellar vesicles with an onion-like structure which have formed the basis of a large amount of research and have given rise to several patents (WO-A-93/19735; WO-A-95/18601; WO-A-97/00623; WO-A-98/02144; WO-A-99/16468). They are distinguished from liposomes by the following:

- their preparation method, which starts from a lamellar phase in thermodynamic equilibrium;
- their internal liquid crystal structure formed by a regular stack of concentric bilayers of amphiphilic compounds alternating with layers of water or an aqueous solution or a solution of a polar liquid (for example glycerol);
- the varied nature of the amphiphilic molecules that can be used to constitute them, used alone or as a mixture.

International patent application WO-A-99/16468 describes vesicles with an onion-like structure incorporating an antigen therein and which can amplify the immune response to that antigen. The examples of that International patent application, which for the first time describes incorporation (also termed encapsulation in that document) of antigens in a multilamellar vesicle with an onion-like structure, clearly show that such encapsulation can substantially amplify the immune response during parenteral administration of an antigen encapsulated in a multilamellar vesicle with an onion-like structure.

The present inventors have now discovered that administration via the mucosa, in particular nasal administration, of compositions of the type described in WO-A-99/16468, causes an immune response not only in different mucous sites but also in the systemic compartment. This response is characterized by immunoglobulin A (IgA) induction. Such a result is not at all obvious having regard to WO-A-99/16468 since, under conditions in which injection was carried out in the examples of that application, even in the encapsulated form, the antigen did not induce any detectable IgA response.

Immunoglobulins (Ig) or antibodies (Ab) associated with cellular components are essential protagonists in the immune response directed against a pathogen. Igs are proteins that are highly specific as regards the antigen.

Different classes of antibodies have been classified, which are distinguished by their mode of induction, their localization and their function (recognition, neutralization of toxic activity or of enzymatic activity).

In the blood compartment, the majority of Ig produced is circulating IgG (sub-divided into different sub-classes), IgA and IgE remaining very much in the minority. In contrast, the major isotype in the mucosa

is IgA, which is produced by IgA plasmocytes of the mucous lymphoid system and secreted actively by the epithelium of the mucosa.

IgA are specialized antibodies and are adapted to defending mucous surfaces that are constantly exposed to pathogens. They are adapted since, because of their biochemical structure (glycosylation, polymerization, secretory piece), they resist the effects of proteases secreted by the micro-organisms and since, in contrast to other isotypes, they limit inflammatory reactions in those compartments in a state of constant activation. They are specialized since they act on a number of levels: secreted, they bind pathogens, limit the effects of toxins and the entry of pathogens by neutralization; they can also act on the epithelium during their transcytosis, or in the lamina propria. Thus, they represent an essential active barrier which, associated with non specific mechanisms, limit invasion by pathogens.

Naturally, cytotoxic and specific responses combine with the antibody response to eliminate the pathogen. If the local response is not sufficient, a systemic response is triggered.

One highly interesting characteristic of the mucous immune system is the re-circulation of B and T lymphocytes induced at a mucous site and their possible domiciliation in sites other than the induction site, propagating the specific response to other tracts. This phenomenon is very important as regards vaccination.

While the mechanisms for induction of a mucous immune response are still in the course of being elucidated, it often remains extremely difficult to induce a mucous response by parenteral administration of an antigen (with or without adjuvant) and even by mucous administration of the antigen. In fact, parenteral administration of an antigen leads to the induction of specific circulating antibodies of the IgG type. This

type of injection cannot induce IgA in the mucous (nor the systemic) compartments. It is very clear that parenteral vaccination will not reinforce the natural local defenses required to combat many respiratory or genital infections, for example.

For these reasons, it is completely surprising even in the light of the teaching of WO-A-99/16468 that the same antigen incorporated in the same vesicles, administered via the mucosa (nasally) can induce not only a systemic response (IgG in serum) confirming the preceding results by another route, but also a response at mucous sites. Incorporation of the antigen into vesicles with an onion-like structure causes the production of IgA in the compartment linked to the administration site (bronchio-pulmonary) but also in other mucous compartments with a predominance in genital secretions. Further, it should be noted that human serum albumin (HSA) which is used by way of example is a very slightly immunogenic antigen which, when administered nasally alone without incorporation into vesicles, is incapable of inducing any response, whether mucous or systemic.

Further, it should be noted that, compared with parenteral administration, administration via the mucosa necessitates loading the antigen into the mucosa and thus penetration of the antigen, which implies that the antigen must be presented in an optimal manner to defeat the non specific defense mechanisms (movement of cilia, mucus) and to resist the enzymes present in the mucosa or on its surface. It appears that these parameters are optimized by the structure of the vesicles during administration via the mucosa. These functions of vesicles in administration via the mucosa are not necessarily present during parenteral administration, where injection directly reaches zones more favorable to capture by cells of the immune system.

The result observed by administration via the mucosa is thus not a simple extrapolation of the result obtained by parenteral administration, but a demonstration of a novel characteristic of vesicles with an onion-like structure.

The present inventors have now discovered that vesicles identical to those described in International application WO-A-99/16468 incorporating an antigen cause an immune response that is much stronger than administering the free antigen. Further, for certain antigens that induce no response by mucous administration, administering them incorporated into multilamellar vesicles with an onion structure can induce a remarkable response. Finally, the induced response is found not only locally in the mucous compartment (mucous response), but also in the blood circulation (systemic seric response).

Furthermore, these multilamellar vesicles are prepared from biocompatible constituents that are known to be innocuous. Still further, the preparation method is simple to carry out and requires only routinely used chemical apparatus. The fact that the process uses a lamellar phase initially in thermodynamic equilibrium endows it with excellent reproducibility, and the vesicles obtained are highly stable.

In a first essential characteristic, the invention concerns the use of multilamellar vesicles with an onion-like structure having an internal liquid crystal structure formed by a stack of concentric bilayers based on amphiphilic agents alternating with layers of water, an aqueous solution or a solution of a polar liquid and into which at least one antigen is incorporated, for the production of a composition, more particularly a pharmaceutical composition, in particular a vaccine composition, for administration via the mucosa.

The term "incorporated", the use of which appears to us to be preferable to that of the term "encapsulated",

means that the antigen or antigens form an integral part of the entity constituted by the vesicle. In fact, molecules of antigen(s) can be found in any layer between the center and the periphery of said vesicle.

As will become clear from the following detailed description and examples, the pharmaceutical compositions used in the invention allow the preparation of a mucous vaccine intended to induce a mucous response and/or a systemic seric response.

As will become clear from the examples, when they are administered via the mucosa, the vesicles with an onion-like structure as defined above incorporating an antigen induce the production of antibodies in man and in animals.

As indicated above, one advantage of the invention is to induce a very high production of antibodies characterized by the presence of IgA and IgG. This implies an increase in the frequency of lymphocytes carrying IgA and IgG specific to the antigen. The preparation can thus be used for the purposes of activation and differentiation of antigen-specific B lymphocytes that can then be used in cell fusion to produce monoclonal antibodies. In fact, specific lymphocytes present in large quantities in nodes draining the administration site can be immortalized by fusion with a non secreting myeloma and result in hybridomas secreting monoclonal antibodies.

Its capacity to simplify and increase the antibody response means that the invention can also be employed for the production of antibodies, in particular polyclonal IgA, an isotype that is difficult to generate in conventional operational modes, or polyclonal IgG. These antibodies can be used for non therapeutic purposes, for example for research, more particularly for biological or immunological research.

Antibody sampling and purification methods are known to the skilled person.

Thus, the invention also concerns a method for producing antibodies, more particularly IgA.

Further, it has been shown that the composition of the invention, when administered via the mucosa, induces a protection of the organism to the infection for which the antigen incorporated into said composition is responsible.

In a further essential feature, the invention concerns a method for treating a human or animal body by vaccination via the mucosa, in which a composition is administered that contains multilamellar vesicles with an onion-like structure having an internal liquid crystal structure formed by a stack of concentric bilayers based on amphiphilic agents alternating with layers of water, an aqueous solution or a solution of a polar liquid and into which at least one antigen is incorporated.

As will become apparent from the following description and examples, it has been shown that when administered via the mucosa, the compositions of the invention in which the antigen is incorporated into a vesicle with an onion-like structure as defined above can cause a mucous immune response and stimulate the common mucous lymphoid tissue. Such capacities have been shown to be of particular importance regarding antigens with a vaccinating potential as opposed to invasive pathogens with mucous tropism.

The dual possibility of inducing both a mucous response and a systemic response is of huge interest in vaccination as it simplifies administration while offering immunity on a number of fronts: mucous to defend against micro-organism access, and systemic to defend against more disseminated or generalized infections.

Further, the results obtained in the context of the invention demonstrate that the invention can not only amplify the antibody response but can also generate an immune response with a protective efficacy against

infection. Therefore, the invention is applicable both to the production of antibodies and to vaccination.

The results obtained confirm that vesicles administered via the mucosa, in particular nasally, can be used to induce or amplify the antibody response in the mucous compartments, to disseminate this response to other sites more distant from the administration site, and generate a systemic response.

The diameter of the vesicles used in the invention is generally in the range 0.1 micrometers (μm) to 25 μm, preferably in the range 0.2 μm to 15 μm.

More precisely, the vesicles used in accordance with the invention are preferably constituted by a plurality of layers of amphiphilic agents alternating with layers of aqueous or polar phase. The thickness of each of these layers is molecular, typically of the order of 5 nanometers (nm) to 10 nm. For a stack of ten to a few tens of layers, then, a diameter is obtained that is in the range from 0.1 μm to a few tens of micrometers. This has been observed experimentally, the vesicles being observable under optical microscopes (in polarized light to provide a better contrast because of their birefringence), either as unresolved points for the smallest layers, or as birefringent spheres for the largest. The size profile can be studied using a laser granulometer (using static laser beam diffusion, analyzed under a plurality of angles). In general, a gaussian profile is obtained centered on a value of 0.1 μm to 25 μm, showing a slight heterogeneity of size for a given formulation, under the given operating conditions for the preparation.

As described above, vesicles into which the antigen is incorporated have a multilamellar onion-like structure and are constituted, from their center to their periphery, by a succession of lamellar layers separated by a liquid medium. These vesicles can be obtained by a method comprising preparing a lamellar liquid crystal

phase and its transformation by applying shear. Such a method has been described in particular in WO-A-93/19735 claiming priority from French patent FR-A-2 689 418 or WO-A-95/18601, hereby incorporated by reference.

In French patent FR-A-2 689 418, this transformation can be carried out during a homogeneous shear step for the liquid crystal phase, which produces vesicles that are also known as microcapsules, with a controlled size. However, adjusting the formulation of the lamellar liquid crystal phase, in particular the nature of the surfactants forming part of its composition, can enable to transform this liquid crystal phase into vesicles by simple mechanical stress, in particular when mixing the constituents.

Such vesicles also have the advantage, inter alia, of being capable of being prepared by a particularly simple preparation method that can employ a wide variety of surfactants.

A further advantage, also essentially linked to the method used to prepare the vesicles with an onion-like structure used in accordance with the invention, lies in the fact that the active ingredients and additives are incorporated prior to forming the vesicles, which provides an excellent encapsulation yield, and therefore better efficacy, and also economizes on very expensive molecules.

Such structures are advantageously obtained by incorporating at least one antigen into a lamellar liquid crystal phase comprising at least one surfactant then transforming this lamellar liquid crystal phase into a dense phase of small multilamellar vesicles.

Thus, the vesicles used in the invention can be obtained using a process in which a liquid crystal lamellar phase incorporating at least one antigen is prepared and said liquid crystal phase is caused to rearrange itself into multilamellar vesicles by applying shear.

This shear can be homogeneous shear, with the advantage of resulting in perfectly homogeneous vesicles. However, simple mechanical agitation can prove sufficient to result in the formation of the multilamellar vesicles of the invention.

The antigen can be any molecule against which an immune response is desired, whether exogenous such as an infectious pathogenic organism, parasite or micro-organism (yeast, fungus, bacteria or virus), or of intrinsic natural origin (auto-immune disease or cancer). It may have different biochemical natures.

In particular, it may be an antigen selected from the group consisting of :

- proteins, in particular extracted or recombinant proteins, which may or may not be glycosylated;
- peptides;
- lipopeptides;
- polysaccharides;

or is a mixture of a plurality of these components.

The multilamellar vesicles with an onion-like structure are prepared using methods described above, in particular in International patent application WO-A-99/16468. The amphiphilic molecules used to prepare them will be selected, but this is not obligatory, from molecules that are described in the pharmacopeia, or are already used in drugs applied to the mucosa.

According to an advantageous embodiment, the membranes of the vesicles contained in the compositions of the invention contain at least one surfactant selected from the group consisting of :

- phospholipids, which may or may not be hydrogenated;
- linear or branched, saturated or mono- or poly-unsaturated C_6 to C_{30} fatty acids, in the form of

an acid or of a salt of an alkali or alkaline-earth metal, or of an amine;

- esters, which may or may not be ethoxylated, of said fatty acids and
  - saccharose;
  - sorbitan;
  - mannitol;
  - glycerol or polyglycerol;
  - glycol;
- mono-, di- or tri-glycerides or mixtures of glycerides of said fatty acids;
- linear or branched, saturated or mono- or poly-unsaturated C_6 to C_{30} fatty alcohols, which may or may not be ethoxylated;
- ethers, which may or may not be ethoxylated, of said fatty alcohols and:
  - saccharose;
  - sorbitan;
  - mannitol;
  - glycerol or polyglycerol;
  - glycol;
- polyethoxylated vegetable oils, which may or may not be hydrogenated;
- block polymers of polyoxyethylene and polyoxypropylene (poloxamers);
- polyethyleneglycol hydroxystearate;
- alcohols with a sterol skeleton such as cholesterol or sistosterol;
- sphyngolipids;
- polyalkylglucosides;
- copolymers of polyethylene glycol and alkylglycol (for example the ELFACOS family from AKZO NOBEL);
- di- or tri-block copolymers of ethers of polyethyleneglycol and polyalkyleneglycol (for example the ARLACELL family from ICI).

Optional co-surfactants can be added to these surfactants (which can be used alone or as a mixture), to

improve the rigidity and tightness of the membranes forming the vesicle. Examples of such molecules that can be cited are:

- cholesterol and its derivatives, in particular charged or neutral cholesterol esters such as cholesterol sulfate;
- other derivatives with a sterol skeleton, in particular those of plant origin (sitosterol, sigmasterol,...);
- ceramides.

The formulation can advantageously involve a mixture of surfactant molecules. In general, at least two different surfactants are used with different hydrophilic-lipophilic balances, which allows continuous adjustment of the bilayer properties and can control the appearance of an instability, which governs the formation of the multilamellar vesicles.

Advantageously, from the above surfactants, two surfactants are selected with relatively different properties, in particular a different hydrophilic-lipophilic balance (HLB). The first surfactant will advantageously have a hydrophilic-lipophilic balance in the range 1 to 6, preferably in the range 1 to 4, while the second surfactant will have a hydrophilic-lipophilic balance in the range 3 to 15, preferably in the range 5 to 15.

The preparation obtained after transformation of the lamellar liquid crystal phase into multilamellar vesicles can then be diluted, in particular with an aqueous solvent such as a buffer solution, a saline solution or a physiological solution, for example, to obtain an aqueous suspension of vesicles.

The encapsulation technique used in the present invention can readily achieve very high encapsulation yields, which may be as high as 100%. However, such yields are not always vital to the function of the envisaged applications.

The encapsulation yield of the antigen(s) in the compositions of the invention is advantageously more than 50%, preferably more than 80%.

It appears that the structure of the vesicles is responsible for the particularly advantageous results obtained, and that the multilamellar vesicles of the invention allow the antigen to arrive intact at the antigen presenting cells (APC) and assist its capture by these cells. It thus appears that the function of the vesicles of the invention is to vectorize, protect and improve capture of the antigen by the immune system.

A further advantage of the technology is that natural or artificial polymers can be added to this formulation, such as polysaccharides (alginates, chitosan, etc.) to reinforce the solidity of the vesicle, and to enable it to remain longer at the administration site or in the organism, thereby delivering the antigen over a longer period. These polymers can either be incorporated into the vesicle, or be deposited around it in the form of a coating. In this case, the diameter of the vesicle or particle formed from vesicles coated with the polymer matrix is greater than that of the vesicles alone. These polymers can optionally be cross-linked to further reinforce their solidity.

Furthermore, and this constitutes a further advantage of the technology, the formulation can be completed by adding immuno-modulating molecules (chitosan, interleukines, ...), which have intrinsic properties that reinforce amplification and orientation of the immune response.

Vesicles incorporating antigens are advantageously prepared in a process consisting of preparing the lamellar phase in a first stage. This is obtained simply by mixing the ingredients, in an order determined by the scientist according to the miscibilities of each of the constituents. It may be necessary to heat certain pasty

or solid constituents to facilitate their incorporation. In that case, the antigen is preferably added when mixing is complete to avoid subjecting it to too high a temperature. It is also possible to prepare a mixture of all of the constituents except for the antigen or its aqueous solution in the form of a stock mixture, which is used as required to prepare the lamellar phase. The aqueous solution can contain different constituents that ensure its biological compatibility, in particular the buffer mixtures but also the different antigens. The lamellar phase so prepared is then subjected to moderate shear (0 to 1000 s^{-1}) for a limited period (0 to 60 minutes).

In the majority of cases, shear is obtained directly by the action of the device performing the mixing. For very small quantities, it can be obtained by hand by mixing the preparation using a microspatula in an Eppendorf type tube.

The sheared lamellar phase is then dispersed in a final medium, in general water or a buffer, which may be identical to that used during the preparation of the lamellar phase. This dispersion is advantageously produced at ambient temperature (20°C-25°C) by slowly adding the medium to the lamellar phase with constant stirring.

A preservative and optional other additives intended to complete the galenical formulation can be added to the product.

All of the compositions described above comprising at least one antigen incorporated into vesicles with a lamellar onion-like structure have the advantage of being capable of being used for administration via the mucosa and in particular nasally and induce a mucous and/or seric systemic response.

Example I below clearly demonstrates such an effect with HSA.

Figures 1 and 2 accompanying this example summarize the results obtained from different mucous samples (Figure 1) and from animal serum (Figure 2) after nasal administration of the compositions of the invention (group I) compared with those obtained by administration either of compositions in which the same antigen is free (group II) or compositions containing the same vesicles, but empty (group III).

Example II illustrates a process for immunization with FHA. The results obtained in this example are illustrated in Figures 3, 4 and 5, which respectively show:

- Figure 3: antibody responses in different mucous sites;
- Figure 4: antibody responses in serum;
- Figure 5: the pulmonary bacterial load in mice infected with *Bordetella pertussis*.

EXAMPLES

EXAMPLE 1: PRODUCTION OF HSA-SPECIFIC ANTIBODIES

I - Preparation of vesicles containing human serum albumin (HSA)

Formulation (percentage by weight)

1. Potassium oleate (FLUKA):	5.0%
2. Lauric alcohol, ethoxylated with 4 ethylene oxides (SEPPIC):	2.0%
3. Lanolin cholesterol (FLUKA):	5.0%
4. Cholesterol 3-sulfate (SIGMA):	2.5%
5. PBS 1x, sterile (LIFE TECHNOLOGIES):	20.0%
6. Phospholipon 90G soya lecithin (NATTERMANN):	45.5%
7. Human albumin (SIGMA), with 30 mg/ml in PBS 1x:	20.0%

Operating procedure

The components were sterilized with UV irradiation for 60 minutes. The containers and accessories (spatulas, stirrers...) were flame sterilized immediately prior to use.

Constituents 1 to 5 are introduced into a pill maker in no particular order then heated to 80°C for 60 minutes

with very vigorous magnetic stirring. Total dissolution of constituents 3 and 4 was verified microscopically.

The desired quantities of mixture 1 to 5 were introduced into a sterile 1.5 ml Eppendorf tube at ambient temperature then constituents 6 and 7 were added. The ensemble was homogenized using a sterile needle then left overnight at 4°C.

The preparation was then dispersed to 33.33% in sterile PBS 1x.

II- Immunization protocol

To test the effect of the vesicles of the invention during administration via the mucosa, 6-8 week old BALB/c female mice received, nasally, twice (at D0 and D30), the different preparations described below. Nasal administration necessitated anaesthesia of the animals by a solution, a mixture of Ketamine, Valium and Atropine, injected intraperitoneally. One month after the last immunization, the animals were sacrificed, the serum was collected and samples were taken of the mucous secretions with the exception of the vaginal lavages, which were taken from the live mouse over the three days preceding the end of the experiment.

Immunization groups

The mice were divided into 4 groups, I to IV, group IV constituting a control group (non immunized mice, also known as naïve mice) and the other groups being subjected to an immunization protocol as defined above using the following products:

- Group I:
- Encapsulated HSA: 20 µl, per nostril, of the vesicles of the invention incorporating HSA, corresponding to 80 µg of HSA per mouse;
- Group II:
- HSA: 20 µl, per nostril, of the vesicles of the invention incorporating HSA, corresponding to 80 µg of HSA per mouse;

- Group III:

- Empty vesicles: 20 µl, per nostril, of empty vesicles of the invention.

Secretion sampling

All samples were taken with cold solutions (4°C) and were placed in ice as they were taken to limit degradation by proteolytic enzymes.

Bronchio-alveolar lavages:

The mouse trachea was cannulated using a probe and 750 µl of PBS was injected slowly to prevent a hemorrhage, the lungs were washed 3 times with the same solution, and the sample was then centrifuged to eliminate pulmonary cells and separated into aliquots that were stored at -20°C until assay.

Vaginal lavages

These samples were taken from the non-anaesthetized live mouse by injecting 50 µl of PBS into the vaginal orifice and washing the vagina three times with the same solution. This sample was taken over three consecutive days to cover variations in the hormonal cycle of the mouse. The secretions were combined and stored at -20°C.

Intestinal lavages

The intestine was removed, freed from the mesentera and rinsed with water to eliminate external blood. It was then cut longitudinally and incubated in ice in 1 ml of a lavage solution enriched with protease inhibitor. The whole was centrifuged and the supernatant was recovered and frozen at -20°C.

Antibody assay

HSA-specific antibodies present in the serum and secretions were assayed using ELISA to determine the HSA specific IgA and IgG (biotinylated anti-IgA/streptavidine peroxidase, anti-IgG peroxidase). The results are expressed as the mean titer determined with respect to a reference serum from the naïve mouse and corresponding to

the inverse of the dilution equal to a reference threshold.

III - Results

The results of the specific antibody assay are shown in the form of two figures (Figure 1 and Figure 2) respectively illustrating the antibody responses obtained in mucous samples for the response associated with the mucosa and in animal serum (systemic response).

In these figures, in each case we also indicated the number of mice that reacted to the immunization protocol with respect to the number of mice subjected to this protocol (the mention "n/m" signifying that n mice reacted out of m mice subjected to the immunization protocol).

It is clear from these two figures that nasal administration of the vesicles of the invention incorporating HSA cause a substantial production of antibodies in the lungs (Figure 1). Only immunization in the encapsulated form causes a pulmonary immune response of isotype IgA and IgG (the pulmonary existence of these two isotypes has been reported in various publications). While this administration route is not very easy in an animal, all of the animals immunized nasally with the vesicles of the invention responded.

Analysis of the other mucous samples revealed the presence of HSA specific IgA in the vagina and intestine of animals immunized by the vesicles of the invention incorporating HSA, which mucosa were very distant from the administration site, with a predilection for vaginal mucosa. These responses indicate a generalization of the response induced in the pulmonary or nasal mucous lymphoid tissue and the circulation and re-distribution of activated and differentiated lymphocytes close to the administration site.

In contrast to the pulmonary mucosa, the predominant HSA specific isotype in the intestine and vagina was IgA. While IgG_{H+L} titers could not be carried out for the

vaginal lavages, similar experiments carried out with another antigen indicated the predominance of IgA in vaginal secretions.

Only the vectorized form of the antigen resulted in an intense and specific response. The few animals presenting a detectable IgA response in the groups immunized with free HSA (titer of 3 as opposed to 650 in the vaginal secretions with vesicles of the invention containing HSA) could not be differentiated from the group using empty vesicles of the invention.

Studying the animal serum (Figure 2) indicates that the vesicles of the invention incorporating nasally administered HSA are capable of causing a systemic response characterized by the major presence of IgG but also a large quantity of seric IgA. Only the vectorized form led to this production (IgA, HSA alone = 35; IgA, vesicles of the invention HSA = 17620). It is remarkable to obtain a systemic response to the vectorized antigen. It should be remembered that it is extremely difficult to induce a seric IgA response by systemic immunization with an antigen alone or accompanied by an adjuvant authorized for human use.

In conclusion, not only does the encapsulated form cause a pulmonary immune response but also, only this form permits dissemination of the induced response in the respiratory tract to other mucosa, indicating that the vesicles of the invention are powerful vectors for inducing mucous immunity, encouraging loading and induction of the response at a site and re-distributing it to other sites.

EXAMPLE II: PRODUCTION OF FHA-SPECIFIC ANTIBODIES AND PROTECTION AGAINST *BORDETELLA PERTUSSIS*

FHA protein is a filamentous adhesin of *Bordetella pertussis*, the whooping-cough bacterium. In contrast to the HSA used above, FHA is more immunogenic and is thus susceptible of itself inducing an antibody response. This protein forms part of protective antigens contained

in commercial vaccines against whooping-cough. Finally, the murine model can be infected with *Bordetella pertussis* and it is thereby possible to carry out an infection test following mucous administration and to demonstrate the protective nature of the response induced by immunization.

A - Antibody induction

I - Preparation of vesicles containing FHA

Formulation

1. Potassium oleate (FLUKA):	5.0%
2. Lauryl ether of PEG-4 (Simulsol P4, SEPPIC):	2.0%
3. Lanolin cholesterol (FLUKA):	5.0%
4. Cholesterol 3-sulfate (SIGMA):	2.5%
5. PBS 1x, sterile (LIFE TECHNOLOGIES):	20.0%
6. Soya lecithin (Phospholipon 90G NATTERMANN):	45.5%
7. FHA from *Bordetella pertussis* in PBS (1 mg/ml):	20.0%

The operating protocol for the preparation was similar to that of Example 1. The FHA concentration of the finished product was 3 µg for 40 µl.

II - Immunization protocol

To study the antibody response, the mucous immunization and sampling protocol was identical to that described in Example 1. Two groups of 5 animals were constituted, group I receiving the antigen encapsulated in the vesicles, and group II receiving the non encapsulated antigen in solution in PBS. For each immunization, each animal received 3 µg of FHA, distributed into two instillations, one into each nostril.

Secretion sampling

The samples were taken in a manner that was strictly identical to that described for Example 1.

Antibody assay

The FHA specific antibodies present in the serum and secretions were assayed using ELISA using a protocol identical to that described in Example 1, with reagents specific to the FHA antigen.

III - Results

The results are presented in Figures 3 and 4, showing the mean titers of IgA and IgG antibodies in mucous secretions (Figure 3) and in the serum (Figure 4) for the two groups I and II.

The mucous secretions were respectively studied as described in Example 1, from bronchio-alveolar lavages (A), intestinal lavages (B) and vaginal lavages (C).

In group I, mice immunized by the antigen incorporated into the vesicles of the invention (Figure 3), all of the animals responded to the antigen while when the free antigen was administered, only 1/5 of the animals responded, and then very weakly.

As was the case with HSA in Example 1, a very strong amplification of the antibody response in the mucous sites was observed. Nasal immunization with the antigen incorporated into the vesicles of the invention enables to induce a mucous response (IgA) in the lungs, close to the administration site (IgA titer for an antigen incorporated into the vesicles of the invention, 8925, compared with 4.25 for the free antigen), but also a mucous response disseminated into other secretory compartments (intestine and vagina) (titer of vaginal IgA, 13500 for the antigen incorporated into the vesicles of the invention compared with 2.5 for the free antigen). Further, IgG were detected in significant amounts in the three mucous compartments being studied.

As was the case with HSA, we observed large titers of IgA and IgG antibodies in the circulation (Figure 4) following nasal administration of the antigen incorporated into the vesicles (IgA titer for the antigen in the vesicles of the invention, 1300 compared with 39.2 for the free antigen; IgG titer 1 265 000 compared with 3 000).

The results of this Example 2 completely confirm those obtained for HSA. They confirm that administered nasally vesicles can be used to induce or amplify the

antibody response in mucous compartments, to disseminate this response to other sites more distant from the administration site and to generate a systemic response.

B - Protection test

To check whether the immunization obtained was protective, a protection test known as a challenge test was carried out. In this experiment, immunized mice were infected nasally with a fixed amount of *Bordetella pertussis*, then the animals were sacrificed at different times, to observe the progress of infection. The measurement was made by counting the colonies in the lungs.

I - Preparation of vesicles containing FHA

The formulation for and preparation of the vesicles were strictly identical to that used to characterize the antibody response.

II - Immunization protocol

The immunization protocol and the doses of administered antigens were identical to those used to induce antibody.

Three groups of mice were used:

I. mice immunized by the antigen incorporated into the vesicles of the invention;

II. mice immunized by the free antigen in solution in PBS;

III. non-immunized mice.

Each group was composed of 4 mice, immunized at the same time, with the same batch of antigen, in order to carry out the same infection measurements under the same conditions.

III - Infection protocol

Four weeks after the second immunization, the mice were anaesthetized and infected with *Bordetella pertussis* nasally (5×10^7 bacteria/mouse, administered in 20 µl) and the initial infectious load was verified from 3 hours after infection.

IV - Protocol for measuring the infectious charge

The mice were sacrificed and their lungs were removed at different times after infection and dilacerated in PBS to obtain a homogeneous suspension. Different dilutions of the suspension were spread on specific nutrient medium for Bordetella and the colonies were counted after growing for 3 days.

V - Results

The results are shown in Figure 5, which indicates the number of colonies of *Bordetella pertussis* in the lungs of mice 3 days (D3, solid bar) and 5 days (D5, open bar) after infection, in millions of bacteria colonies for the three groups of mice shown below:

Group I: mice immunized with antigen incorporated into the vesicles of the invention.

Group II: mice immunized with free antigen in solution in PBS.

Group III: non-immunized mice.

It can be seen that, from the third day following infection, the bacterial load for mice immunized with the antigen incorporated into the vesicles of the invention was lower (factor 2) than that for non immunized mice (factor 8). Remarkably, while 5 days after infection, the mice immunized with the free antigen and the non-immunized mice experienced the bacterial load increase, the mice immunized with the antigen incorporated into the vesicles of the invention had fewer bacteria on D5 than on D3, indicating favorable evolution of the disease. Further, at this stage, the number of bacterial colonies in the group immunized with the antigen incorporated into the vesicles of the invention was 6 times lower than the group immunized with free FHA and 13 times lower than in the non immunized group, indicating good protection.

These results demonstrate that the invention can not only amplify the antibody response, but that it also generates an immune response with protective efficacy

against infection. The invention is thus also applicable to the production of antibodies and to vaccination.

CLAIMS

1. Use of multilamellar vesicles with an onion-like structure having an internal liquid crystal structure formed by a stack of concentric bilayers based on amphiphilic agents alternating with layers of water, an aqueous solution or a solution of a polar liquid and into which at least one antigen is incorporated, for the production of a composition for administration via the mucosa.

2. Use according to claim 1, characterized in that said composition is a composition intended to induce a mucous response.

3. Use according to claim 1 or claim 2, characterized in that said composition is a composition intended to induce a systemic seric response.

4. Use according to any one of claims 1 to 3, characterized in that said composition is a composition intended to induce the production of antibodies.

5. Use according to any one of claims 1 to 4, characterized in that said composition is a pharmaceutical composition, in particular a vaccine, intended to induce protection of the organism against the infection for which said antigen is responsible.

6. Use according to any one of claims 1 to 5, characterized in that said antigen is of exogenous or intrinsic natural origin.

7. Use according to any one of claims 1 to 6, characterized in that said antigen is selected from the group consisting of :

- proteins, in particular extracted or recombinant proteins, which may or may not be glycosylated;

- peptides;
- lipopeptides;
- polysaccharides;

or is a mixture of a plurality of these components.

8. Use according to any one of claims 1 to 7, characterized in that said vesicles contain at least one surfactant selected from the group consisting of :

- phospholipids, which may or may not be hydrogenated;
- linear or branched, saturated or mono- or poly-unsaturated C_6 to C_{30} fatty acids, in the form of an acid or of a salt of an alkali or alkaline-earth metal, or of an amine;
- esters, which may or may not be ethoxylated, of said fatty acids and
  - saccharose;
  - sorbitan;
  - mannitol;
  - glycerol or polyglycerol;
  - glycol;
- mono-, di- or triglycerides or mixtures of glycerides of said fatty acids;
- linear or branched, saturated or mono- or poly-unsaturated C_6 to C_{30} fatty alcohols, which may or may not be ethoxylated;
- ethers, which may or may not be ethoxylated, of said fatty alcohols and:
  - saccharose;
  - sorbitan;
  - mannitol;
  - glycerol or polyglycerol;
  - glycol;
- polyethoxylated vegetable oils, which may or may not be hydrogenated;

- block polymers of polyoxyethylene and polyoxypropylene (poloxamers);
- polyethyleneglycol hydroxystearate;
- alcohols with a sterol skeleton such as cholesterol or sistosterol;
- sphyngolipids;
- polyalkylglucosides;
- copolymers of polyethylene glycol and alkylglycol;
- di- or tri-block copolymers of ethers of polyethyleneglycol and polyalkyleneglycol.

9. Use according to any one of claims 1 to 8, characterized in that said vesicles also contain at least one co-surfactant intended to improve the rigidity and/or tightness of the membranes of said vesicles.

10. Use according to claim 9, characterized in that said co-surfactant is selected from the group consisting of formed by:

- cholesterol and its derivatives, in particular charged or neutral cholesterol esters such as cholesterol sulfate;
- derivatives with a sterol skeleton, in particular those of plant origin such as sitosterol or sigmasterol;
- ceramides.

11. Use according to any one of claims 1 to 10, characterized in that said vesicles also contain an immuno-modulating substance.

12. Use according to any one of claims 1 to 11, characterized in that the diameter of said vesicles is in the range 0.1 μm to 25 μm, preferably in the range 0.2 μm to 15 μm.

13. Use according to any one of claims 1 to 12, characterized in that the bilayers of said vesicles comprise at least two surfactants, one of which has a hydrophilic-lipophilic balance (HLB) in the range 1 to 6, preferably in the range 1 to 4, and the other has a hydrophilic-lipophilic balance (HLB) in the range 3 to 15, preferably in the range 5 to 15.

14. Use according to any one of claims 1 to 13, characterized in that the encapsulation yield of the antigen (or antigens) in said vesicles is more than 50%, preferably more than 80%.

15. Use according to any one of claims 1 to 14, characterized in that said administration via the mucosa is nasal administration.

16. A method of treating a human or animal body by vaccination, characterized in that it comprises administration via the mucosa of a composition as defined in any one of claims 1 to 15.

17. A method according to claim 16, characterized in that said administration is carried out nasally.

18. A method of producing antibodies, characterized in that it comprises introducing into a host organism, via the mucosa, lamellar vesicles with an onion-like structure having an internal liquid crystal structure formed by a stack of concentric bilayers based on amphiphilic agents alternating with layers of water, an aqueous solution or a solution of a polar liquid and into which at least one antigen is incorporated, then removing and purifying said antibodies.

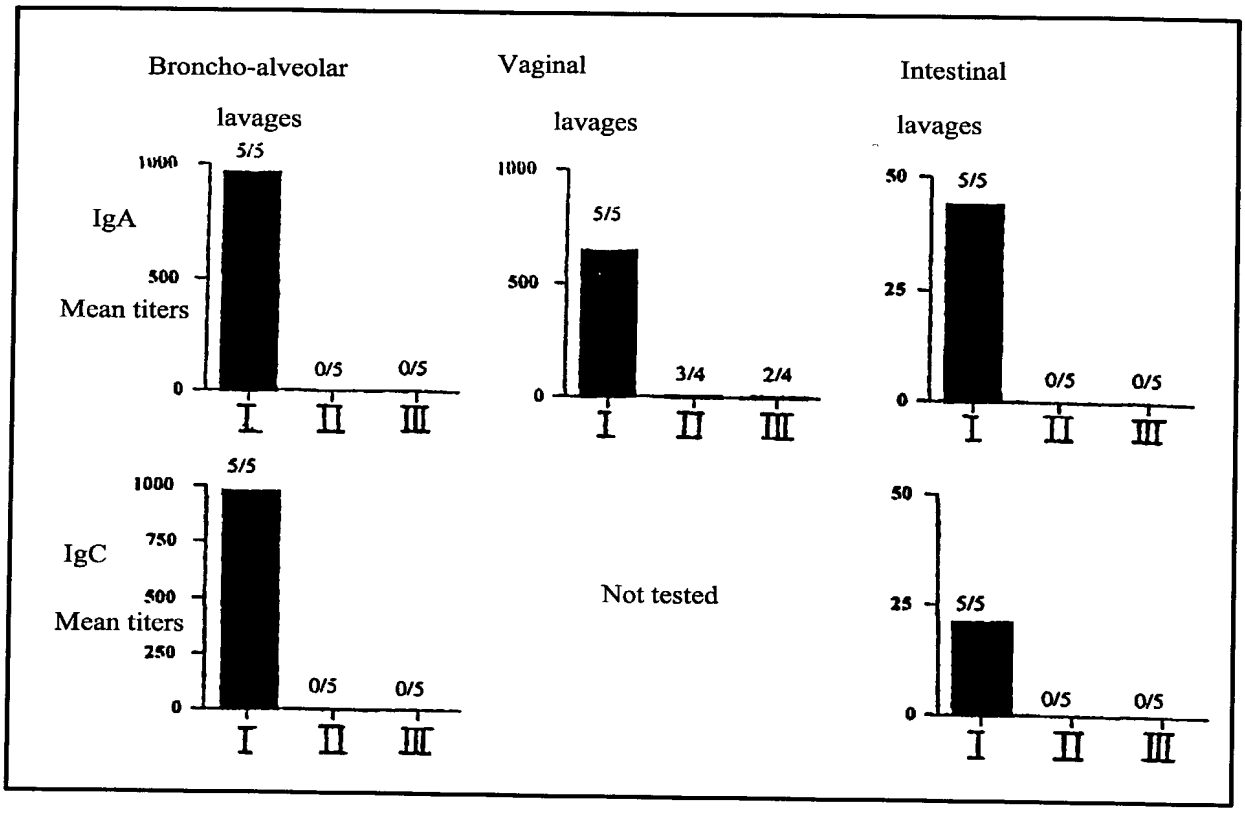
19. A method of producing IgA, characterized in that it comprises introducing into a host organism, via the

mucosa, lamellar vesicles with a multilamellar onion-like structure with an internal liquid crystal structure formed by a stack of concentric bilayers based on amphiphilic agents alternating with layers of water, an aqueous solution or a solution of a polar liquid and into which the appropriate antigen is incorporated, and removing and purifying said immunoglobulins.

A B S T R A C T

COMPOSITION TO BE ADMINISTERED THROUGH MUCOUS MEMBRANE

The present invention relates to a novel use of multilamellar vesicles with an onion-like structure having an internal liquid crystal structure formed by a stack of concentric bilayers based on amphiphilic agents alternating with layers of water, an aqueous solution or a solution of a polar liquid and into which at least one antigen is incorporated, for the production of a composition, more particularly a pharmaceutical composition, in particular a vaccine composition, for administration via the mucosa, to induce a mucous response and/or a seric systemic response and/or to protect the organism against an infection for which said antigen is responsible. The invention also relates to a method for vaccination via the mucosa and a method for producing antibody, in particular IgA. More particularly, the invention is applicable to nasal administration.


Broncho-alveolar
lavages
IgA
Mean titers
1000
500
0
5/5
0/5
0/5
I
II
III
IgC
Mean titers
1000
750
500
250
0
5/5
0/5
0/5
Vaginal
lavages
1000
500
0
5/5
3/4
2/4
Not tested
Intestinal
lavages
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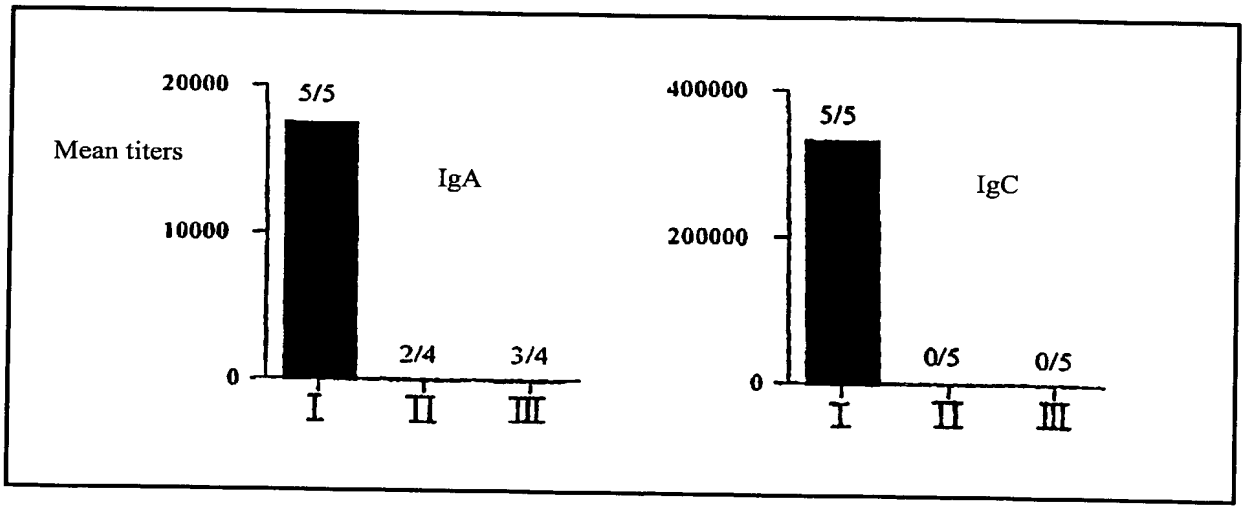
5/5
0/5
0/5
50
25
0
5/5
0/5
0/5

FIG.1


Mean titers
20000
10000
0
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2/4
3/4
IgA
400000
200000
0
5/5
0/5
0/5
IgC

FIG.2

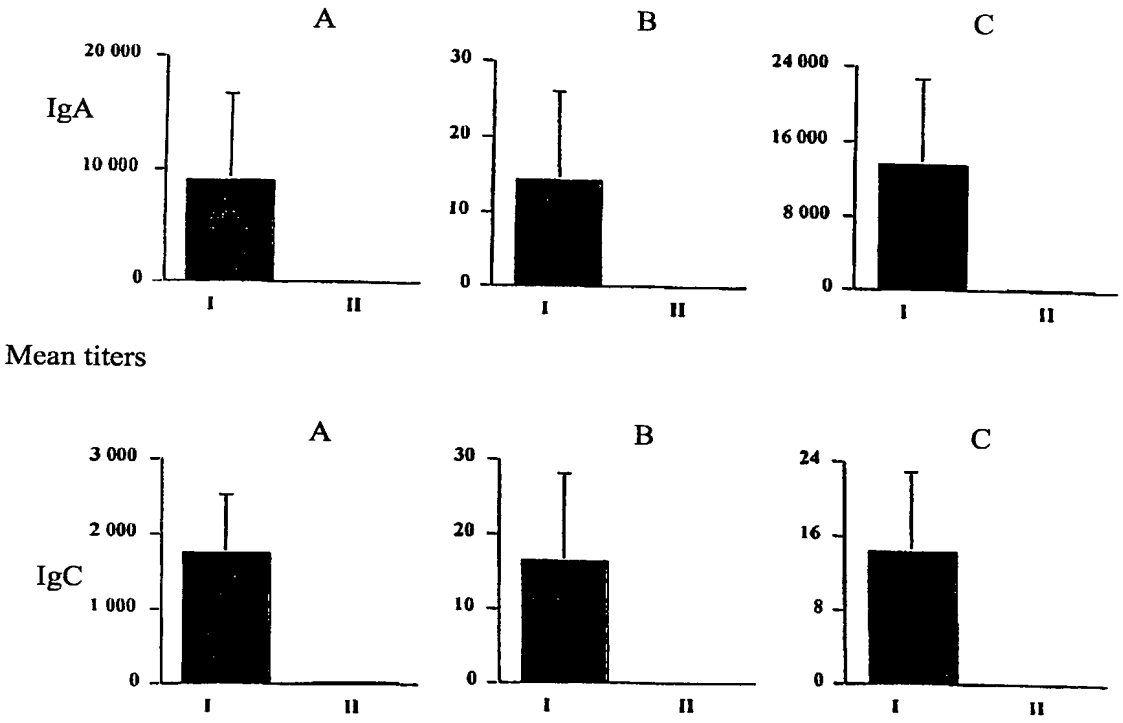

A
B
C
20 000
IgA
10 000
0
I
II
30
20
10
0
I
II
24 000
16 000
8 000
0
I
II
Mean titers
A
B
C
3 000
2 000
IgC
1 000
0
I
II
30
20
10
0
I
II
24
16
8
0
I
II

FIG.3

FIG.4

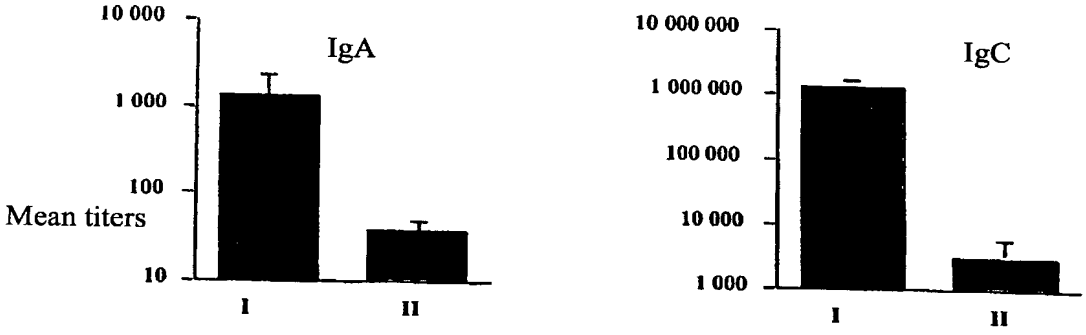
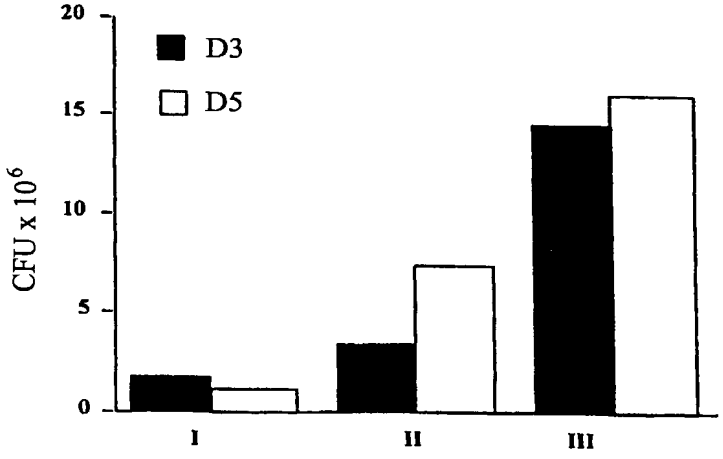

IgA
10 000
1 000
100
Mean titers
10
I
II
IgC
10 000 000
1 000 000
100 000
10 000
1 000
I
II

FIG.5


D3
D5
CFU x 10^6
20
15
10
5
0
I
II
III

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY'S DOCKET NUMBER

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

COMPOSITION TO BE ADMINISTERED THROUGH MUCOUS MEMBRANE

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application

Serial No. _____

on _____,

and was amended

on _____ (if applicable).

☒ was filed as PCT international application

Number PCT/FR00/02523

on SEPTEMBER 13, 2000,

and was amended under PCT Article 19

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowlege the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT, indicate PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
FRANCE	99/11465	September 14, 1999	☒ YES ☐ NO
			☐ YES ☐ NO
			☐ YES ☐ NO
			☐ YES ☐ NO
			☐ YES ☐ NO

1005-0855 Nga Ca-14

Combined Declaration For Patent Application and Power of Attorney (Continued)
(Includes Reference to PCT International Applications)

ATTORNEY S DOCKET NUMBER

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowlege the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S APPLICATIONS		STATUS *(Check one)*		
U.S. APPLICATION NUMBER	U S FILING DATE	PATENTED	PENDING	ABANDONED
PCT APPLICATIONS DESIGNATING THE U S				
PCT APPLICATION NO	PCT FILING DATE	U S SERIAL NUMBERS ASSIGNED *(if any)*		

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(List name and registration number)*

Donald L. Dennison Reg.No. 19920
Burton Scheiner Reg.No. 24018
Ira J. Schultz Reg.No. 28666
Scott T. Wakeman Reg.No. 37750

Send Correspondence to: DENNISON, SCHEINER, SCHULTZ & WAKEMAN
1745 Jefferson Davis Highway, Suite 621
Arlington, Virginia 22202-3417

Direct Telephone Calls to: *(name and telephone number)*
(703) 412-1155
(703) 412-1161 (fax)

201	FULL NAME OF INVENTOR	FAMILY NAME: GAUBERT	FIRST GIVEN NAME: Sophie	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY: PESSAC FR	STATE OR FOREIGN COUNTRY: FRANCE	COUNTRY OF CITIZENSHIP: FRANCE
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: Résidence les Cottages de la Réserve, App16, 14 rue des	CITY: As/	STATE & ZIP CODE/COUNTRY: 33600 /France
202	FULL NAME OF INVENTOR	FAMILY NAME: LAVERSANNE	FIRST GIVEN NAME: René	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY: PESSAC FR	STATE OR FOREIGN COUNTRY: FRANCE	COUNTRY OF CITIZENSHIP: FRANCE
	POST OFFICE ADDRESS	POST OFFICE ADDRESS: 62 avenue du Parc d'Espagne	CITY: PESSAC	STATE & ZIP CODE/COUNTRY: 33600/FRANCE
203	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201	SIGNATURE OF INVENTOR 202	SIGNATURE OF INVENTOR 203
Sophie Gaubert		
DATE: February 25, 2002	DATE: February 25, 2002	DATE